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Award Number:
W81XWH-08-1-0158

TITLE:
ROLES FOR THE DNA DAMAGE CHECKPOINT PROTEIN HUS1 IN BREAST CANCER

PRINCIPAL INVESTIGATOR:
Stephanie Yazinski

CONTRACTING ORGANIZATION:
Cornell University
Ithaca, NY 14850

REPORT DATE:
July 2009

TYPE OF REPORT:
ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
07-31-09	Annual	1 Jul 2008 - 30 Jun 2009

4. TITLE AND SUBTITLE
ROLES FOR THE DNA DAMAGE CHECKPOINT PROTEIN HUS1 IN BREAST CANCER

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0158

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stephanie A. Yazinski

Email: say5@cornell.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Cornell University
Ithaca, NY 14850

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and and Materiel
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Cancer is aberrant, uncontrolled cellular proliferation arising from an accumulation of mutations in growth regulatory genes. Two mammalian DNA damage checkpoint pathways, the Atm and Atr pathways, act to suppress tumor formation by preventing mutation accumulation and inducing senescence in response to oncogenic stimuli. Roles for the Atr pathway in tumor suppression are less understood, as deletion of any member of this pathway, including Hus1, results in embryonic lethality. To understand roles for Hus1 in breast cancer suppression, we developed mouse models featuring partial Hus1 impairment and are testing how Hus1 dysfunction affects cellular responses to activated oncogenes. Focus formation assays revealed that cultured cells with reduced Hus1 levels develop fewer transformed foci than control cells. Additionally, population doubling analyses and anchorage-independent growth assays with cells expressing activated oncogenes will be performed. To elucidate roles for Hus1 as a tumor suppressor in vivo, mice expressing reduced levels of Hus1 were crossed to mice overexpressing ErbB2 in the mammary gland to generate a cohort of mice. Our preliminary results indicate that reduced Hus1 levels may decrease the capacity of cells to undergo transformation, suggesting that Hus1, or the Atr pathway, may be a possible target for breast cancer treatment.

15. SUBJECT TERMS
Breast cancer, Hus1, Genomic instability, Checkpoint, Oncogene

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction:

Cancer arises from aberrant, uncontrolled cellular proliferation due to an accumulation of mutations in growth regulatory genes (1). An increase in the rate of mutation accumulation, termed genomic instability, can drive tumor formation and is particularly important in the genesis of breast cancer, as defects in several genes that normally function to preserve genomic integrity cause increased breast cancer risk (2). DNA damage checkpoints are a key genome maintenance mechanism and suppress tumor formation by preventing mutation accumulation and also by triggering senescence in response to oncogenic stimuli. The two primary mammalian checkpoint pathways center on the large protein kinases, Atm and Atr (3). Mutations in the better-characterized Atm pathway are well known to confer an increased risk of many cancers, including breast cancer (2, 4). The Atr pathway, by contrast, is essential for organismal viability and thus is far less well understood, but nevertheless has been hypothesized to function in tumor suppression (5-7). Hus1, an essential member of the Atr pathway, is a component of the Rad9-Rad1-Hus1 (9-1-1) heterotrimeric, PCNA-like sliding clamp, which is recruited to sites of DNA damage and is necessary for optimal phosphorylation of the Atr target and checkpoint effector Chk1 (8). In order to study the effects of Hus1 dysfunction while bypassing the embryonic lethality associated with complete Hus1 inactivation, our lab developed an allelic series which expresses incrementally reduced levels of Hus1 (9). The objective of this project is to use the Hus1 allelic series to determine how partial Hus1 impairment impacts neoplastic transformation in cultured cells and mammary tumorigenesis in mice. This study will provide critical new insights into how checkpoint dysfunction, and specifically the Atr pathway, influences breast carcinogenesis and will establish new breast cancer mouse models that may be of great use for evaluating therapeutics.

Body:

Task 1. Determine the effects of reduced Hus1 levels on cell transformation by activated oncogenes

a. Cell proliferation assay: Immortalized and primary mouse embryonic fibroblasts (MEFs) from the Hus1 allelic series are being maintained in normal and low oxygen conditions. These cells will be infected with virus expressing GFP, activated H-Ras, or both activated H-Ras and c-Myc. These viral vectors for expression of H-Ras as well as H-Ras and c-Myc have been obtained and transfected into viral packaging cells. The resulting viruses will be used to infect cells which will then be passed and counted every two days for twenty-four days. Population doublings will be calculated as a measure of cell proliferation.
b. Focus formation assay: Primary and immortalized MEFs from the Hus1 allelic series were transfected with expression plasmids encoding activated H-Ras, activated H-Ras and E1A, or E1A alone and tested for formation of transformed cell foci. In both primary and immortalized cultures, MEFs expressing the lowest level of Hus1 showed fewer foci than control MEFs following transfection with activated H-Ras and E1A (Fig. 1). This indicates that cells with reduced levels of Hus1 are less prone to neoplastic transformation following oncogenic signaling, resulting in a reduced number of focus formation. Primary MEFs with the lowest

level of Hus1 expression do not grow well in normoxia due to sensitivity to reactive oxygen species, which may result in reduced focus formation. To overcome this, our lab purchased a low oxygen incubator which will be used to assess the focus formation in primary MEFs from the Hus1 allelic series.

c. Anchorage independent growth assay: Immortalized and primary MEFs from the Hus1 allelic series are being maintained in normal and low oxygen conditions. These cells will be infected with virus expressing GFP, activated H-Ras, or both activated H-Ras and c-Myc. The resulting viruses will be used to infect immortalized MEFs from the Hus1 allelic series in order to produce cell pools that will be grown in soft agar to determine anchorage independent growth potential, another indicator of malignant transformation.

Task 2. Determine the effects of reduced Hus1 levels of on mammary tumorigenesis in transgenic mice expressing activated oncogenes

a. Mice expressing varying levels of Hus1 were interbred with transgenic mice expressing activated H-Ras in the mammary gland. These mice developed mammary and Harderian gland tumors very quickly, within 4 weeks. Because of this short tumor latency, these mice could not be readily used for interbreeding to generate a tumor cohort. For this reason, we concluded that MMTV-Ras would not be a useful strain to determine the effects of reduced Hus1 levels on mammary tumorigenesis in mice, and instead have focused on another mouse mammary tumor model as described below.
b. Mice expressing varying levels of Hus1 and transgenic mice overexpressing ErbB2 in the mammary gland (MMTV-Neu) were interbred to generate a cohort of mice (Table 1).
c. Monitor tumor formation: The oldest of these mice have been aged for one year, with two control ($Hus1^+$ MMTV-Neu^+) mice developing mammary tumors, while their female littermates with reduced levels of Hus1 have not developed tumors yet.
d. Histopathological analysis of mammary glands: The first mouse to develop a mammary tumor was euthanized when the tumor had grown to 2cm in diameter. This tumor was fixed and paraffin embedded, and will be analyzed by H&E staining, as well as TUNEL staining, Ki67 staining, and stained for markers of senescence.

Key Research Accomplishments:

- Viral vectors expressing oncogenes, activated H-Ras and both H-Ras and c-Myc, have been transfected into virus packaging cell lines to produce replication-defective recombinant viruses.
- Primary and immortalized cells from the Hus1 allelic series have been maintained in normoxia as well as low oxygen conditions and will be used to determine the effects of reduced Hus1 levels on cell transformation by activated oncogenes.
- Focus formation assays have shown that cells with reduced levels of Hus1 develop fewer foci following transfection with two oncogenes, suggesting that the reduced levels of Hus1 interfere with neoplastic proliferation.

- A cohort of MMTV-Neu expressing mice consisting of an equal number of mice with reduced levels of Hus1 and control ($Hus1^{+}$) mice, are aging, and are being monitored for mammary tumorigenesis (Table 1).
- The first mouse to develop a mammary tumor was euthanized, and the tumor was fixed and paraffin embed for future histopathological analysis.

Reportable Outcomes:

Meeting Attended:

Breast Cancer and Environmental Risk Factors, Regional Cancer and Environment Forum, Rochester, NY, May 2008.

Meeting Presentations:

Chromosomal Instability and p53-indepent Apoptosis Following Conditional Inactivation of the DNA Damage Checkpoint Gene Hus1 in the Mouse Mammary Gland. Stephanie Yazinski, Peter Westcott, Kelly Ong, Jan Pinkas, Rachel Peters, Robert Weiss. AACR Meeting: Mouse Models of Cancer, San Francisco, CA. January 2009. (poster presented)

A Partial Defect in the Checkpoint Protein Hus1 Impairs Tumor Development in a Two-Step Skin Carcinogenesis Model. Stephanie Yazinski, Lee Gerwitz, Tiffany Shand, Rachel Peters, Robert Weiss. Twelfth Annual Buffalo DNA Replication and Repair Symposium. May 2009 (poster presented)

Combined inactivation of Hus1 and p53 in the mouse mammary gland results in accumulation of damaged cells and impaired tissue regeneration. Stephanie Yazinski, Peter Westcott, Kelly Ong, Jan Pinkas, Rachel Peters, Robert Weiss. Twelfth Annual Buffalo DNA Replication and Repair Symposium. May 2009 (presentation given)

Manuscript:

Dual inactivation of Hus1 and p53 in the mouse mammary gland results in accumulation of damaged cells and impaired tissue regeneration. Stephanie Yazinski, Peter Westcott, Kelly Ong, Jan Pinkas, Rachel Peters, Robert Weiss. (in revision).

Conclusions

Focus formation assays in both primary and immortalized cells have been performed. These assays revealed that cells with reduced levels of Hus1 develop fewer transformed foci following transfection with two oncogenes. This suggests that cells with reduced levels of Hus1 are more resistant to malignant transformation than cells with at least one copy of wildtype Hus1. Hus1 may be required for cell survival following the stress of neoplastic proliferation. This suggests that reducing the levels of

Hus1 may prevent cells with activated oncogenes from being fully transformed and that Hus1 or the Atr pathway may be exploited as a target for cancer therapy.

All the reagents to perform viral transductions, including the viral DNA constructs and the packaging cells, have been acquired and prepared. This will allow for transduction of both primary and immortalized cells with oncogenes in order to perform anchorage independent growth assays and population doubling assays. These assays will further establish if reduced levels of Hus1 decrease the susceptibility of cells to malignant transformation.

Finally, a cohort of mice with decreasing levels of Hus1 which overexpress an oncogene specifically in the mammary gland have been generated, are aging, and have begun developing mammary tumors. These mice will be useful in determining if reduced levels of Hus1 can reduce transformation and tumorigenesis *in vivo*, as is seen in cell culture experiments.

Taken together, preliminary data from foci formation assays suggest that cells require greater than 20% of wild-type Hus1 levels in order to efficiently undergo transformation. These results will be further tested in other cell culture assays and in an in vivo mouse breast cancer model. The results of these studies will show whether Hus1, or the Atr pathway, may be a possible drug target for treatment of breast cancers.

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Supporting Data:

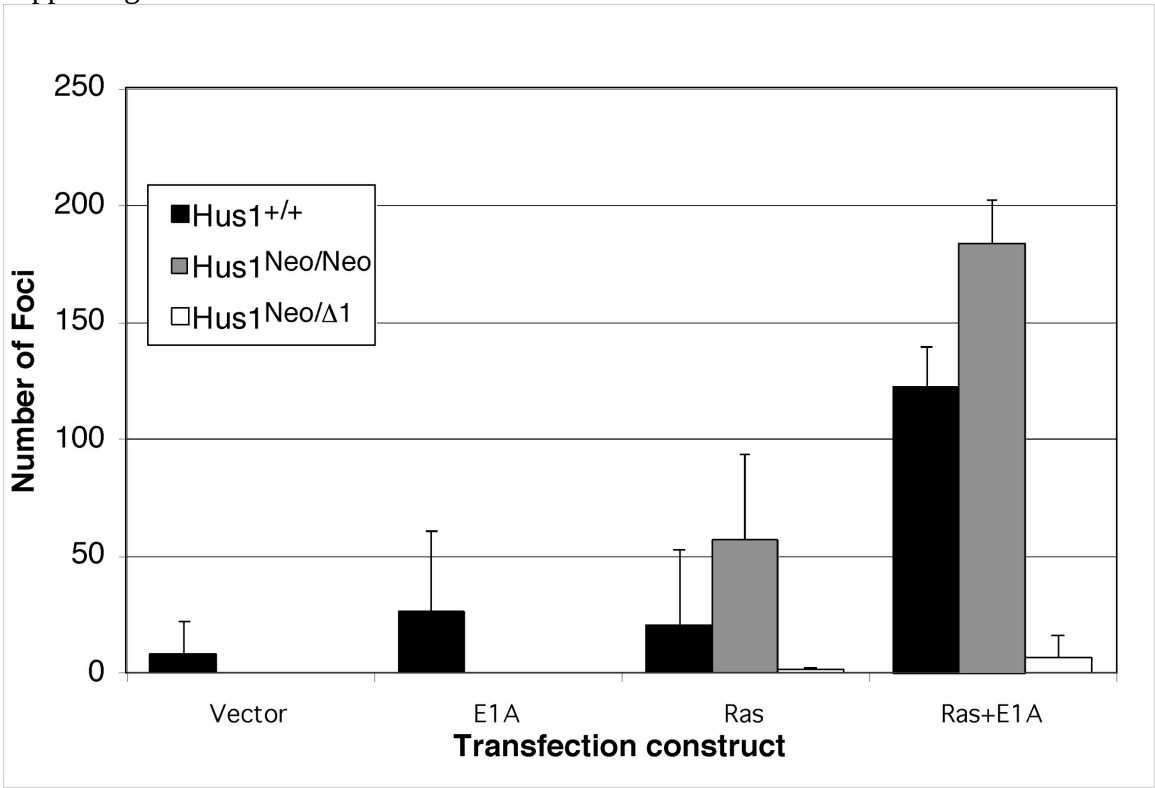

Figure 1: Immortalized MEFs expressing wildtype Hus1 (Hus1+/+), ~40% Hus1 ($Hus1^{Neo/Neo}$), or ~20% Hus1 ($Hus1^{Neo/\Delta1}$) were transfected with activated oncogenes, Ras, E1A, or Ras and E1A, in a focus formation assay. MEFs expressing the lowest levels of Hus1 developed fewer foci when compared to controls, suggesting a decreased propensity of cells with severely impaired Hus1 expression to undergo transformation. However, $Hus1^{Neo/Neo}$ cells, which have a less severe reduction of Hus1 expression, showed an increase in focus formation, suggesting that moderately impaired Hus1 results in increased transformation.

Genotype	Number of Animals	Number with Tumors
$Hus1^{+}$ MMTV-Neu^{+}	35	2
$Hus1^{Neo/\Delta 1}$ MMTV-Neu^{+}	22	0
$Hus1^{+}$ MMTV-Neu^{-}	5	0
$Hus1^{Neo/\Delta 1}$ MMTV-Neu^{-}	7	0

Table 1: Mice expressing varying levels of Hus1 and transgenic mice overexpressing ErbB2 in the mammary gland (MMTV-Neu) were interbred to generate a cohort of mice that will be used to determine the effect of reduced Hus1 expression on mammary tumor development. $Hus1^{Neo/\Delta 1}$ MMTV-Neu^{+} mice express the lowest levels of Hus1 in the Hus1 allelic series. $Hus1^{+}$ MMTV-Neu^{+} mice serve as controls for mammary tumor development. Additionally, $Hus1^{+}$ MMTV-Neu^{-} and $Hus1^{Neo/\Delta 1}$ MMTV-Neu^{-} mice were generated as negative controls. The table lists the number of mice for each genotype in the cohort as well as the number of animals that have developed tumors to date.